

Mast Cells Are Early Responders After Hypoxia-Ischemia in Immature Rat Brain

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Background and Purpose—Perinatal hypoxia-ischemia (HI) produces acute and prolonged inflammation of the brain. Mast cells (MCs), numerous in the pia and CNS of neonatal rats, can initiate inflammation attributable to preformed mediators. MCs contribute to HI brain damage in the neonatal rat; MC stabilization protects through 48 hours of reperfusion. Here we hypothesize that HI induces early MC migration, activation, and release of proinflammatory molecules.

Methods—HI was induced by right CCA ligation and 75 minutes 8% oxygen. Histochemistry and immunocytochemistry described the time course of early cellular changes in the CNS. For neuroprotection by MC stabilization, pups were treated with Cromolyn (CR) during the initial 24 hours post-HI; brains were examined through 4 weeks.

Results—Brain MC number and activation were elevated in ipsilateral hemisphere immediately after HI ($P < 0.05$), before detection of cleaved caspase-3 in neurons (NeuN+; 2 hours post-HI), astroglial activation (GFAP+ with swollen cell body, 4 hours post-HI), or microglial activation (OX42+, 4 hours post-HI). TNF- α -positive MCs were present in a subpopulation of MCs in control animals and the percent of TNF- α MCs increased dramatically ipsilaterally immediately after HI ($P < 0.01$). Microglial TNF- α was evident at 4 hours; endothelial cells had no detectable TNF- α until 48 hours post-HI. Cromolyn prevented MC migration, reduced brain damage/neuronal loss, glial activation, and brain atrophy through 4 weeks of recovery ($P < 0.05$).

Conclusions—MCs are early responders to HI in neonatal brain. MC stabilization provides lasting protection and suggests a new target for therapeutic interventions. (*Stroke*. 2009;40:3107-3112.)

Key Words: TNF- α ■ Cromolyn ■ inflammation ■ neuroprotection

Perinatal hypoxic-ischemic (HI) brain damage is a major cause of acute mortality and chronic neurological morbidity in infants and children.¹ The mechanisms leading to brain damage after perinatal HI are complex and relate to the developmental stage of the brain and the severity of the insult.^{2,3} Inflammation plays an important role in the pathogenesis of damage and represents an important target for therapeutic intervention. Previous studies on inflammatory events focused predominately on microglial activation, expression of proinflammatory mediators, and infiltration of immune inflammatory cells.^{4–6} Recent studies from our laboratory highlighted a novel cell in the inflammatory response to HI in the postnatal day (P) 7 to 8 rat, the brain mast cell (MC).⁷

MCs were originally designated as components of the innate immune response but are now known as highly plastic cells capable of alterations in phenotype (mediator expression) depending on the local environment and state of activation.⁸ MCs are normal residents in the CNS^{9,10} where they are found in close association with cerebral blood vessels during development and adulthood.^{11,12} Brain MC

numbers increase during neonatal development.^{13,14} Our previous study demonstrated significant elevations of MC number and degranulation in association with neuronal injury at 48 hours after HI in the P7 rat.⁷ There is significant astro- and microglial activation by this time, such that we could not address the identity of the “first responder,” which has been assumed to be microglial.¹⁵ The importance of the MC response, however, was further supported by the demonstration that postinsult treatment of pups with the mast cell stabilizer, disodium cromoglycate (Cromolyn), inhibited mast cell migration and degranulation, glial activation, and neuronal death.⁷ Recent studies in adult rats showed that intracerebral injection of Cromolyn reduced the early cerebral edema and neutrophil accumulation after transient middle cerebral artery occlusion (MCAO),¹⁶ as well as inhibited hemorrhage formation after tPa treatment.¹⁷ Early MC activation was suggested to contribute to cerebral histamine accumulation and damage in another neonatal ischemic model, further supporting a role for MCs in neonatal brain injury.¹⁸ Thus we hypothesized that, as observed in peripheral inflammatory responses, MCs are central to the early re-

Received March 5, 2009; final revision received May 4, 2009; accepted May 8, 2009.

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DOI: 10.1161/STROKEAHA.109.549691

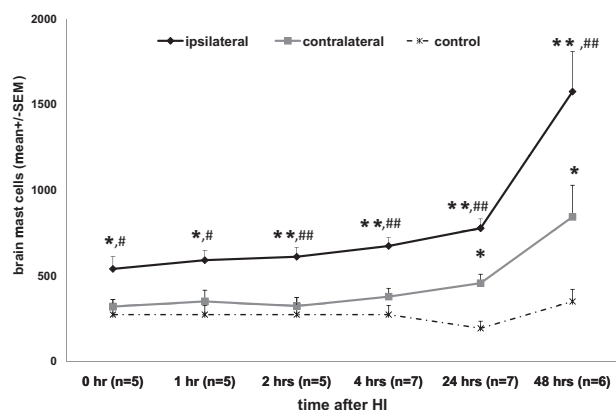


Figure 1. Rapid increase in brain mast cells after hypoxia-ischemia. HI induced a rapid migration of MCs, relative to contralateral or control, which remained elevated through 48 hours. Values are mean numbers of brain mast cells \pm SEM. $**P < 0.01$, $*P < 0.05$ vs control; $\#P < 0.05$, $\#\#P < 0.01$, vs contralateral.

response to HI in the immature brain. To pursue this hypothesis, we investigated the time course of the MC response, relative to other cell types, after moderate HI in the P7 rat, and show that MC activation was the most rapid. In addition, inhibition of immediate MC activation with systemic Cromolyn treatment was sufficient to provide long-term neuroprotection.

Materials and Methods

Animals

Timed pregnant Wistar rats (Charles River, Mass) delivered normally. Unilateral HI was induced in P7 rats according to our standard methodology,^{19,20} with a hypoxic duration of 75 minutes. Nonligated controls were exposed to room air. Cromolyn (Sigma) or saline was administered (50 mg/kg, sc) immediately after, and at 1 and 24 hours. All procedures were approved by the Institutional Animal Care and Use Committee of Columbia University and follow the NIH guidelines.

Tissue Preparation

At 0 hours to 4 weeks after HI, pups were anesthetized with isoflurane and either decapitated (P7-P9) and brains fixed by immersion, or subjected to perfusion with 4% paraformaldehyde. For nonperfused brains chemical fixation was followed by microwave irradiation (80 seconds). Frozen sections (50 μ m) were cut on a sliding microtome (Microm) and collected in sets of 5 sections.

MC Quantification

As previously,⁷ one section per set (1/5) was stained with acidic Toluidine blue (TB, Electron Microscopy Science, PA; pH=2.0) to visualize and quantify MCs.

Neuropathological Analysis

One section per set was stained with hematoxylin and eosin (H&E) and examined by light microscopy (Olympus) with a QImaging CCD camera (QImaging). Morphometric evaluation of damage at 1 to 4 weeks was determined based on the shrinkage of the ipsilateral to contralateral hemisphere.^{21,22} Neuronal damage/death was assessed with activated caspase-3 (1:200, Cell Signaling) and Neuron-specific-Nuclear Protein (NeuN 1:1000, Millipore) and evaluated with confocal fluorescence microscopy (LSM 510, 40 \times objective, laser Argon 488 and Hene 543, Zeiss Inc) or using Fluoro-Jade-B (Chemicon) according to the protocol of Schmued and Hopkins²³ and used previously in our HI experiments.⁷ Sections were examined by fluorescence microscopy (Olympus) with a QImaging CCD

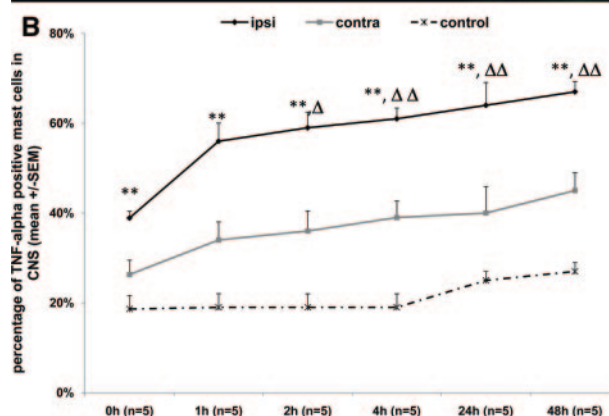
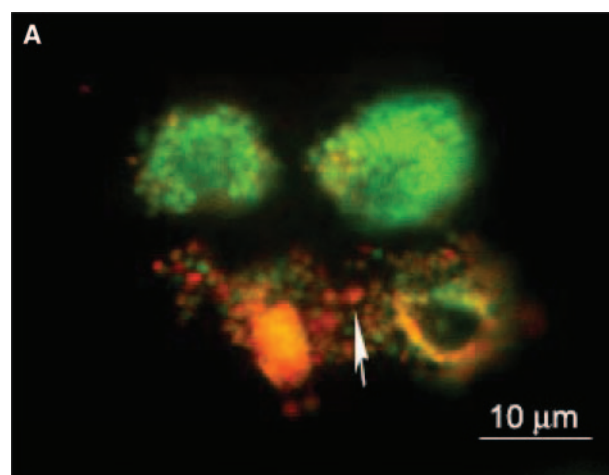


Figure 2. Rapid increase in TNF- α -positive brain mast cells. Double staining for TNF- α (red) and avidin (MCs, green) demonstrates colocalization of this cytokine in brain mast cells. A, TNF- α in MC granules and in granule remnants after exocytosis (arrow). B, The percentage of TNF- α -positive brain mast cells increased in ipsilateral relative to control and contralateral hemisphere. Values represent the percentage of TNF- α -positive brain MCs/total brain mast cells (per brain) \pm SEM. $**P < 0.01$, $*P < 0.05$ vs control; $\Delta P < 0.05$, $\Delta\Delta P < 0.01$, vs contralateral side.

camera (QImaging) using blue light excitation. An ordinal scale of 0 and 4 was established to correspond with the percentage of area damaged/brain region as previously described.⁷

Immunochemistry

Brain sections were incubated in 1 or more of the following primary antibodies at 4 $^{\circ}$ C for 24 to 48 hours: rabbit anti-glia fibrillary acidic protein (GFAP; 1:1000, R. Liem, Columbia University, NY); mouse anti-rat GFAP (1:200, Cell Signaling); mouse anti-rat OX42, a marker for activated microglia/monocytes (1:500, Serotec) and mouse anti-rat RECA -1 for endothelia (1:100, Serotec); goat anti-rat TNF- α (1:50, Pierce). Sections were then incubated (4 $^{\circ}$ C, overnight) in the appropriate fluorescent secondary antibodies (Jackson Labs and Invitrogen) and fluorophore-conjugated egg white avidin (1:600, Sigma), which binds to heparin, a MC specific glycosaminoglycan.²⁴

Quantification of Glial Activation

Using sections stained for GFAP or OX42 antigen, the same ordinal scale as used for estimating the area of FJ positive neurons was used to estimate and compare degree of glial activation under each experimental condition.

Statistical Analysis

Each experimental group consisted 3 to 6 animals. All values were expressed as the mean \pm SEM. One-way ANOVA was used to test for

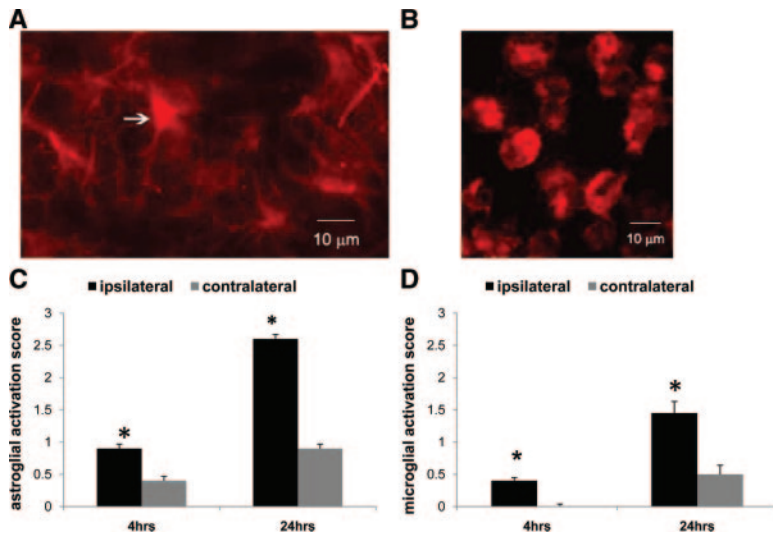


Figure 3. Glial activation after hypoxia-ischemia. Shown are sections from ipsilateral thalamus. A, Activated astrocytes (GFAP+) with swollen cell body (arrow). B, Activated microglia (OX42+) were observed as early as 4 hours post-HI. The astroglial (C) and microglial (D) activation score increased significantly in the ipsilateral, compared to contralateral at 4 and 24 hours post-HI. The bar graph represents mean glial activation score \pm SEM * $P < 0.05$, ** $P < 0.01$.

overall significance followed by the Tukey test as a posthoc test for significance of multiple comparisons ($P < 0.05$).

Results

Time Course of Cellular Responses After HI

To investigate the rapidity of the MC response to HI, relative to the other neural cells, we subjected P7 rat pups to a moderate HI insult and analyzed their brains at 0 to 48 hours of reperfusion. Brain MCs were quantified in toluidine blue (TB) stained sections.⁷ In response to HI, CNS MCs increased rapidly in the ipsilateral hemisphere and were significantly greater than both control and contralateral hemisphere immediately after HI and throughout the initial 48-hour period (Figure 1). The number of brain MCs in the contralateral hemisphere became significantly greater than control only after 24 hours of reperfusion and remained elevated at 48 hours (Figure 1). There was a significant increase in MC number in all animals between P7 and P9, partly reflecting normal developmental influx.

In addition to migration, MC activation and degranulation are necessary to release key mediators such as TNF- α to initiate the inflammatory cascade.²⁵ To identify degranulated and TNF- α -positive MCs, we used fluorescent avidin. Intact and degranulated MCs were observed in both pia and brain parenchyma (Figure 2A). Degranulated cells were characterized by granules/granule-remnants outside of the cell boundary and cytoplasmic regions devoid of granular material.⁷ The percentage of TNF- α -positive MCs in brain increased sharply in the first hour after HI and remained elevated through 48 hours (Figure 2B). TNF- α positive MCs increased in the contralateral hemisphere, albeit more slowly and to a smaller extent. Interestingly, even in control brains, roughly 20% of the resident MCs were TNF- α positive, with a significant increase between P7 and P9, consistent with previous reports that brain MCs contained preformed TNF- α .²⁶

Having established the time course of the early MC response, we evaluated the effect(s) of HI on the other neural cells, including neurons, astrocytes, and microglia, as well as endothelial cells. In the moderate injury paradigm used in these studies, tissue damage evolves over time. Histological

analysis of cell death in H&E sections revealed the presence of pyknotic nuclei, chromatin condensation/fragmentation, and frank tissue loss at select times and regions. The onset of chromatin fragmentation was similar to that of the expression of activated caspase 3. Double labeled (NeuN+) cells were observed as early as 2 hours after HI (supplemental Figure I, available online at <http://stroke.ahajournals.org>). Activated caspase-3-positive neurons increased in number throughout the initial 24-hour period, at which time cell death was evident using Fluoro-Jade B (data not shown, see Jin et al⁷).

Glial cells, astrocytes and microglia, are considered integral to the initiation of the inflammatory response in both immature and adult brains, and activation of both resident microglia and astrocytes is consistently observed after HI. Activated astroglia were characterized by ramifying GFAP+ processes and swollen cell bodies (Figure 3A); activated microglia were characterized by an intense signal for the OX42 antigen (Figure 3B). Astroglial and microglial activation scores are presented in Figure 3C and 3D. Activated glial cells were first detected at 4 hours, with significant increases at 24 hours. Thus, although these cells have routinely been assumed to be the initiators of the post-HI inflammatory response, MC invasion and activation precede glial activation. As activated microglia are also a source of TNF- α , we investigated the time-course of the expression of this cytokine in microglial cells. TNF- α expression was detected in microglia in both the ipsilateral cortex and thalamus at 4 hours (supplemental Figure IIA) and was evident throughout the subsequent 24 to 48 hours. TNF- α expression was delayed in endothelial cells (RECA+) until 48 hours after HI (supplemental Figure IIB). These results support the hypothesis that the mast cells are likely to be the first cells to release TNF- α in response to HI in the immature brain.

Neuroprotection With Cromolyn

If the immediate/early MC activation is necessary for the initiation of the inflammatory cascade and ultimate tissue damage, inhibition of this response should be neuroprotective. We reported previously that cromolyn given at the end of the hypoxic period limited MC migration/degranulation,

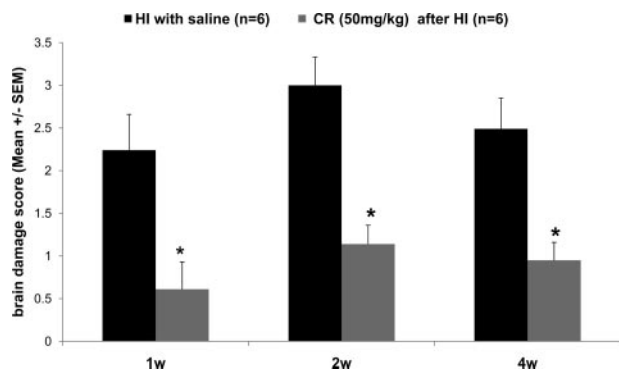


Figure 4. Cromolyn treatment limited brain damage after hypoxia-ischemia. At 1, 2, and 4 weeks post-HI, there was a significant reduction in the brain damage score (measured with Fluoro-Jade B) of HI pups treated with Cromolyn compared to pups injected with saline. The bar graph represents mean brain damage score \pm SEM (* P <0.05).

glial activation, and neuronal loss as assessed at 48 hours.⁷ Here we extended these observations to evaluate long-lasting neuroprotection. Pups received either Cromolyn (50 mg/kg) or saline, sc, at 0, 1, and 24 hours of reperfusion. The half-life of Cromolyn in the human circulation is between 80 to 90 minutes (Merck Manual online); as the last treatment was at 24 hours after HI it is unlikely that Cromolyn was present beyond 48 hours. However, MC stabilization during the initial 24 hours significantly limited further MC migration into the brain and provided a striking degree of neuroprotection assessed by FluoroJadeB damage score (Figure 4) and morphometry through 4 weeks of recovery.

Extensive cerebral atrophy and cystic cavitation are long-term consequences of HI in the P7 rat and were observed in this study at 2 and 4 weeks (Figure 5A). Cavitation was observed in 30% of saline-treated animals at 2 weeks, and 50% at 4 weeks. Cromolyn prevented the loss of brain tissue

(Figure 5B); cavitation was absent from all CR-treated HI brains. The measurement of hemispheric diameters provides an objective quantifiable assessment of the extent of cerebral atrophy/damage in the ipsilateral, relative to the contralateral hemisphere. The extent of neuroprotection attributable to early MC stabilization with Cromolyn translated to normal growth of the ipsilateral hemisphere (Figure 5C) and normal ipsilateral/contralateral ratios (Figure 5D).

Astrocytic and microglial activation was also significantly reduced by Cromolyn at the later time points (Figure 6A and 6B), further supporting our initial hypothesis that it is the early mast cell activation that is responsible for the inflammatory cascade and the subsequent activation of the other cells.

Discussion

Cerebral ischemia induces an inflammatory response in the CNS that may be initiated by the release of cytokines such as TNF- α and IL-1, leading to the recruitment of immune cells, further inflammation, and evolving brain damage.^{27–29} Several studies have investigated the cellular, biochemical, and molecular responses to cerebral ischemia in adult³⁰ and immature nervous systems^{4,5,31} to determine which cells might be the most appropriate targets to inhibit the inflammatory cascade. No previous study has included brain mast cells in this analysis. The current experiments tested 2 hypotheses: (1) MCs are the first cells to respond to HI in immature brain; (2) inhibition of this early MC response is sufficient to provide long-term protection. We observed recruitment and activation of MCs preceded responses of neurons, glia, and endothelial cells by 2 to 4 hours. Subsequently, we inhibited the early MC response with Cromolyn and observed significant neuroprotection through 4 weeks. The results of this study support an important role for early MC activation in HI-induced brain damage and suggest that

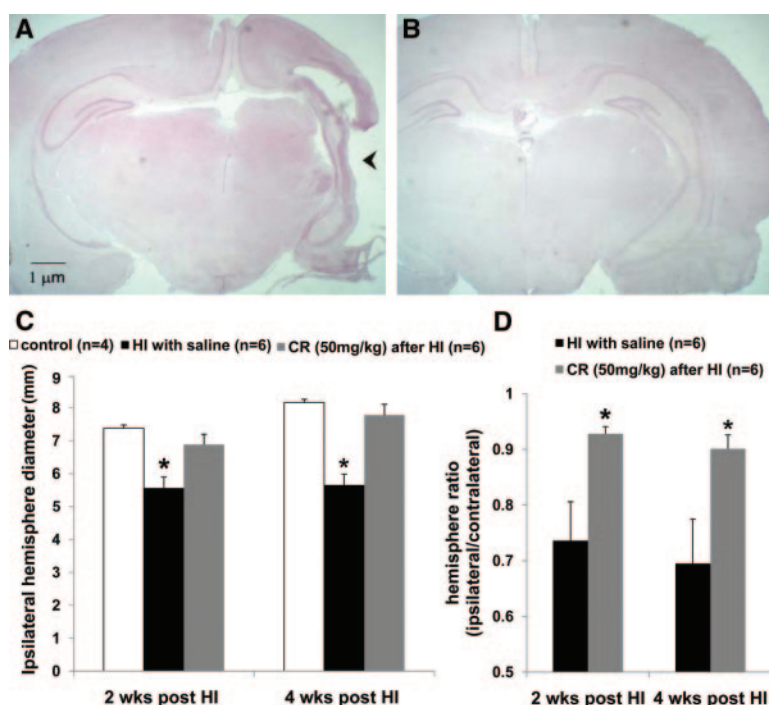


Figure 5. Cromolyn treatment preserved brain maturation after hypoxia-ischemia. H&E staining revealed extensive brain damage at 2 weeks post-HI. Cystic cavitation (arrowhead) in saline-injected animals (A) but not in the brains of the Cromolyn-treated group (B). C, The diameter of the ipsilateral (HI) hemisphere of saline-treated animals was significantly smaller than that of control and Cromolyn-treated HI brains. D, HI reduces the hemispheric ratio; this ratio is near 1 in Cromolyn-treated pups indicating normal growth of the HI hemisphere. Values are mean hemispheric ratio \pm SEM (* P <0.05).

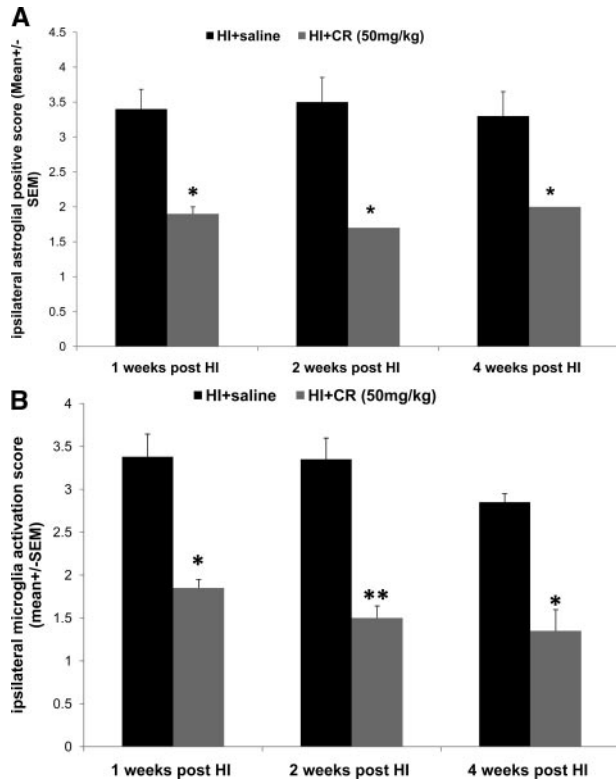


Figure 6. Cromolyn treatment reduced glial activation after hypoxia-ischemia. The inhibition of glial response was observed for both astroglia (A) and microglia (B) measured with GFAP and OX42 immunoreactivity, respectively. Values are mean glial activation score \pm SEM (* $P < 0.05$, ** $P < 0.01$).

inhibition of this response could be a new target for therapeutic intervention in perinatal HI.

TNF- α is a proinflammatory mediator and an increase in TNF- α expression is a consistent component of the acute inflammatory response, as has been implicated in the acute phase of ischemic stroke.³² Clinically, infants with hypoxic-ischemic encephalopathy (HIE) have significantly increased TNF- α in CSF and blood 24 hours after delivery.³³ Bona et al⁴ reported an increase in TNF- α mRNA in both hemispheres at 1 hour of reperfusion after 70-minute HI in the P7 rat, with a peak expression at 12 hours in the ipsilateral hemisphere. Although TNF- α is produced by many cells in response to appropriate stimuli, MCs arrive “armed” to initiate acute inflammation as they, and only they, contain preformed/stored TNF- α ³⁴ after appropriate stimulation.³⁵ Here we observed $\approx 20\%$ of the control brain MCs to be TNF- α positive. HI significantly increased this percentage, resulting in a doubling by the end of the hypoxic interval, with further increases during the first hour of reperfusion; exocytosis of TNF- α granules was evident at the earliest time points. We propose that MCs had also initiated de novo synthesis of TNF- α as the percent of positive MC population was elevated despite the evident degranulation. TNF- α granules were released in the pia where the cytokine could enter the CSF, and directly into the brain parenchyma. Although other resident cells in the CNS produce TNF- α , most notably microglia/macrophages^{36,37} and endothelial cells,³² the pres-

ence and release of this important cytokine from the MCs preceded its detection in other cells.

If MCs are the first responders to HI in the immature brain, what is the signal for their recruitment and activation? A critical role of MC activation in hypoxia and ischemia-reperfusion induced inflammation and injury has been well established in peripheral tissues, and attributed to direct effects of both NO free radicals and reactive oxygen species (reviewed in³⁸). It is possible that initial MC activation is in the pia with release of TNF- α into the CSF and brain parenchyma, thus initiating the inflammatory cascade, and obviating the necessity Cromolyn penetrating the blood-brain-barrier. As Cromolyn is an FDA-approved drug which is currently used in infants and children, it may have a new and valuable clinical application in protection from HIE.

Sources of Funding

This study was funded by a National Institutes of Health grant (R01 MH 54088, to A.J.S.) and an American Heart Association grant (0575055N, to S.J.V.).

Disclosures

None.

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